Methods for Creating Stomatal Impressions Directly onto Archivable Slides

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ABSTRACT

Stomatal density has been shown to be a primary determinant of crop yield, water use efficiency, and limitation to CO_2 assimilation rate. Widely used methods of assessing stomatal density sample relatively small regions of the leaf, are labor intensive, or do not yield stable archivable samples for revisiting samples. We describe several methods of producing such epidermal impressions that yield samples large enough to generate stomatal density maps across entire leaf surfaces.

While morphological factors such as leaf size, shape, trichome density, waxiness, cuticular composition and thickness all affect crop plant gas exchange, the stomata are the primary pathways of CO_2 and water vapor exchange between the leaf interior and the atmosphere. Stomata, as an epidermal feature, have been studied in terms of their functional and evolutionary significance and leaf developmental processes for well over a century (e.g., Salisbury, 1927; Kelly and Beerling, 1995; Edwards et al., 1998; Croxdale, 2000; Raven, 2002; Carpenter, 2005). Along with stomatal architecture and guard cell responsiveness to environmental and physiological cues, stomatal density and distribution have long been recognized as primary determinants of gas conductance through the epidermis (Smith, 1941; Nobel, 1991).

Crop physiologists have long been interested in leaf morphological parameters such as stomatal density and frequency from physiological and developmental perspectives. Stomatal density and conductance affects biomass accretion, yield, assimilation rate, water use efficiency, and canopy cooling. Amphistomy, the presence of stoma on both leaf surfaces, has been suggested as a trait that reduces the resistance to CO_2 uptake under high light or in a plant with thicker leaves (Mott et al., 1982; Mott and Michaelson, 1991). High stomatal density mutants of *Arabidopsis* exhibit enhanced assimilation rates under high light and nonwater-limited conditions (Schlüter et al., 2003). Stomatal density and conductance are strongly correlated in divergent plants such as sorghum [*Sorghum bicolor* (L.) Moench], soybean (*Glycine max* L. Merr.) and *Populus* cultivars (Muchow and Sinclair; 1989; Gitz et al., 2005; Reich, 1984) and increased assimilation rates and yields have been found to be associated with increased stomatal conductance in diverse crops over the past half century (e.g., Cornish et al.,1991; Specht et al., 1999; Faville et al., 1999; Morrison et al., 1999; Jiang et al., 2003). An inadvertent outcome of selecting for yield is that in some cases enhanced stomatal conductance has resulted in similarly enhanced capacity for evaporative leaf cooling and maintenance of photosynthetic capacity at higher temperatures (Radin et al., 1994; Fischer et al., 1998). Selecting for increased stomatal density and conductance has been suggested as a means for enhancing yield in current breeding programs (Richards, 2000). Conversely, reduced stomatal conductance has been postulated as a selection factor through which crop water use efficiency might be manipulated (Condon et al., 2004; Richards et al., 2002; Muchow and Sinclair, 1989).

The techniques used to visualize stomata may be broadly grouped into two classes: the direct observation of fresh or prepared material and the preparation and observation of replicas, or castings, of epidermal features. Direct observation methods include direct viewing of fresh materials under either bright field or fluorescent microscopy (Karabourniotis et al., 2001), teasing the epidermis from the leaf and mounting in water (Eckerson, 1908; Weyers and Travis, 1981), preparing fresh paradermal sections (Gitz and Liu-Gitz, 2003), fixing and sectioning leaf material and viewing under bright field microscopy (Santos et al., 2001), or fixing, freeze drying, and sputter coating to view under scanning electron microscopy. Each of these methods has unique strengths and weaknesses that must be taken into account depending on the species and the experimental goals. For example, attempts to quantify stomatal density of bahiagrass (*Paspalum notatum* var. *notatum* Flügge) by making epidermal replicas (described below) were ineffective because the lower leaf surface was extremely irregular (Santos et al., 2001). So, in that case it was suggested the leaves be fixed, mounted in paraffin, sectioned longitudinally and viewed, taking advantage long parallel rows in which the stoma are arranged. In general, the fixing or sectioning requires considerable labor and expense while allowing only a small amount of surface area to be viewed at a time. Moreover, other methods

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Published in Agron. J. 101:232–236 (2009).
doi:10.2134/agronj2008.0143N

Abbreviations: CA, cellulose acetate; CAB, cellulose acetate butyrate; $MeCl_2$, methylene chloride; MEK, methylethyl ketone; PMMA, polymethyl methacrylate; PVC, polyvinyl chloride.

of direct observation also suffer from disadvantages such as the perishable nature of fresh material and shrinkage of dried material, as well as the commitment of resources to fixing and preparation for microscopic examination.

Two approaches are generally used for viewing replica epidermal features, making peels or making impressions of the leaf surface. Peels are made by applying a low viscosity plastic or resin carried in a volatile solvent to the leaf surface and allowing the liquid to harden. The thin film is then gently peeled from the leaf surface using transparent tape; or fine forceps might be used and the peel subsequently mounted in water. Early methods used collodion, a solution of nitrocellulose in ether (Long and Clements, 1934, and references therein). Since then, a variety of substances have been used such as AcraSeal acrylic spray ignition sealant (Fox, 1970), cellulose acetate solutions (Long and Clements, 1934; Payne 1970), and cyanoacrylate adhesive, or "Superglue" (Wilson, 1981).

Making impressions of the leaf surface is accomplished when the leaf is brought into contact with a viscous gel or a putty which is allowed to harden. Methods include using silicone rubber, agarose gel (Mathur and Koncz, 1997), thin plastic strips softened with solvent (McDonald, 1977) or dental putty (Lawson et al., 1998). The use of dental putty is noteworthy because it is nondestructive, which allows several impressions to be made over time for developmental studies (Berger and Altman, 2000; Geisler et al., 2000). Viewing may then be accomplished via bright field microscopy with transparent replicas, or in the case of opaque replicas, oblique illumination microscopy (Cheng et al., 2002), or by creating a transparent positive replica with epidermal peel techniques described above (Lawson et al., 1998).

While several stomatal morphological characteristics can be quantified such as stomatal aperture, pore diameter, pore depth, and stomatal index; stomatal density is a useful/pragmatic parameter to quantify. Investigation of stomatal density simplifies data analysis since stomatal density is temporally stable as compared to stomatal conductance which varies diurnally or even over the course of minutes. Stomatal density varies across leaf surfaces, so when defining methods to detect differences between plants or treatments it may be necessary to characterize the source of variability by mapping stomatal distribution. (Gitz, 1993; Liu-Gitz et al., 2000; Santos et al., 2001). Here, we describe approaches for producing epidermal impressions which may be directly viewed by bright field microscopy and that can yield samples large enough to generate stomatal density maps of entire leaf surfaces.

METHODS

Clear plastic sheets of various materials and thicknesses were obtained and cut into rectangular pieces equivalent to the size of large commercially available microscope slides (75 × 150 mm). Leaf epidermal impressions were made directly onto 50 × 75 mm slides fashioned from 0.16 mm (1/16 in) cellulose acetate (CA), 4.74 mm (3/16 in) cellulose acetate butyrate (CAB), 2.03 mm (5/64 in) polymethyl methacrylate (PMMA), or 3.17 (1/8 in) clear polished polyvinylchloride (PVC). Solvents (acetone or $MeCl_2$ for CA and CAB, and methylene chloride or methylethylketone for the PMMA and PVC) were applied directly onto a fresh leaf that was sandwiched between two pieces of 4.74 mm (1/16 in) aluminum plate one of which was faced with closed cell Neoprene foam in contact with the leaf. The resulting sandwich was held in place for about 5 min with spring steel clips. The foam was removed and the leaf subsequently peeled from the slide revealing the epidermal impression.

We also investigated using a clear gloss spray laquer (Deft Inc., Irvine CA, www.deftfinishes.com). Leaves were simply taken to the fume hood, both surfaces coated with spray laquer, allowed to dry, given a second coat, allowed to dry, the replicas lifted from the leaf surface with clear urethane packing tape and placed on a plastic 50 × 75 mm slide for viewing.

The resulting epidermal impressions were viewed by bright field microscopy with an Olympus BX60 compound microscope (Olympus Optical Co., Tokyo, Japan).[1] Images were captured with a RT Spot Color digital camera and Spot version 3.5 imaging software (Diagnostic Images Inc., Sterling Heights, MI).

RESULTS

The cellulose acetate method yielded replicas in exquisite detail (see Fig. 1A) but the method suffers from the disadvantage that if an impression is made across the entire surface of the plate, the plate warped within hours so that the impressed surface was slightly concave, making manipulation of the slide with the stage micrometer difficult. However, this problem is not nearly as pronounced as the severe wrinkling observed when 0.127 to 0.254 (5–10 mil) cellulose acetate film was used (not presented). The warping problem was minimized when very small leaves and small amounts of solvent were used for the impressions.

Because of the warping with CA and because we were unable to locate thicker CA plates we opted to use a thicker plate of a related polymer, CAB. The impressions obtained were of similar quality to those made with cellulose acetate (Fig. 1A and 1B) and warping was nonexistent with the plates for a few months. However, after 18 mo in storage some warping was noticed in plates on which the epidermal replica extended across the entire surface. The slight warping did not hinder positioning with the stage micrometer. Again, warping was eliminated when smaller leaflets or leaves were used. To explore the limitations of the system we attempted and were able to obtain an epidermal impression of an entire cotton leaf using an appropriately sized but somewhat thicker aluminum plate (6.35 × 150 × 150 mm). Methylene chloride ($MeCl_2$) and methylethyl ketone (MEK) could also be used with CAB but pieces of leaf tissue tended to be left behind with the softer leaves such as cotton (*Gossypium hirsutum* L.) and tobacco (*Nicotiana tabacum* L.).

While impressions made with the polymethyl methacrylate (PMMA)/$MeCl_2$ system (Fig. 1C) did not yield the detail of the softer CAB system (Fig. 1A), it afforded enough detail to clearly observe stoma in eastern gamagrass [*Tripsacum dactyloides* (L.) L.] leaf. Inspection of Fig. 1E reveals small dark spots arranged in rows across an area of approximately 4 by

[1]Mention of this or other proprietary products is for the convenience of the readers only, and does not constitute endorsement or preferential treatment of these products.

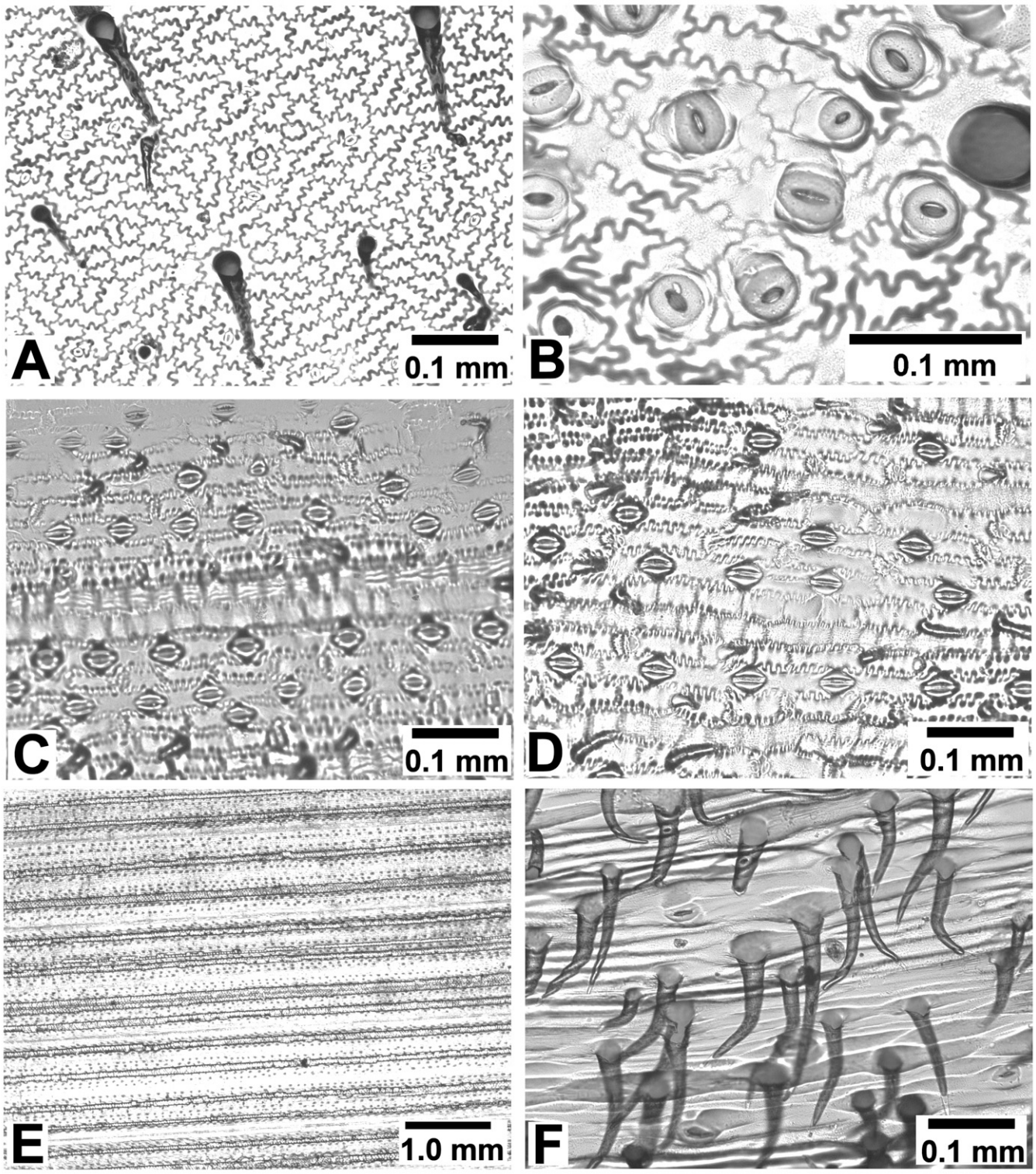

Fig. 1. Brightfield micrographs of epidermal impressions made with various polymer and solvent systems. (A) Cellulose acetate (CA)/acetone impression of adaxial (upper) tobacco leaf epidermis, (B) Cellulose acetate butyrate (CAB)/acetone impression of abaxial (lower) tobacco leaf epidermis, (C) Polymethyl methacrylate (PMMA)/methylene chloride ($MeCl_2$) impression of adaxial sorghum epidermis, (D) CAB/acetone impression of adaxial sorghum epidermis, (E) PMMA/$MeCl_2$ impression of eastern gamagrass epidermis, (F) CAB/acetone impression region around vein of abaxial tomato leaf.

5 mm, which were identified as stoma by viewing at higher magnification. When treated with the solvent, the PMMA was apparently not softened as much as with the CAB acetone. This resulted in large areas of the slide staying within the focal plane when examined (Fig. 1E), but without the detail achievable with the CAB/acetone system (Fig. 1F). Even though details were not resolved as well as with CAB and CA, thinner slides could be made without warping.

While impressions could be made with clear polished PVC plate and MEK, they were of rather low resolution as compared to the CAB/acetone system. It was rather difficult to visualize epidermal features with bright field microscopy (not presented).

Because the images obtained with spray laquer were unremarkable and very similar to those described elsewhere with clear fingernail varnish, they are not presented here. However, although the impressions made were very similar to results obtained using nail polish, impressions of large areas along an entire sorghum leaf could easily be produced and were very easily lifted from the leaf surface. Removing such epidermal peels from the surface of tobacco leaves could be accomplished, but was more difficult to achieve.

DISCUSSION

While we describe novel approaches to producing epidermal impressions across relatively large areas of leaf surface, it should be kept in mind that the principles of these procedures are simply extensions of long established techniques. Chemically, some of the stomatal impressions systems in use today are actually variants of chemical component systems, which have existed for over a century. In the late 1800s and 1900s, techniques were described where collodion, a solution of cellulose nitrate in ether, was applied to the leaf surface, allowed to dry, carefully teased from the surface, and mounted and viewed under a microscope (Long and Clements, 1934, and references therein). Later in the century a widely available household adhesive Duco (ITW Performance Polymers, Riviera Beach, FL, www.itwconsumer.com) houshold cement (Williams, 1973) was recommended for making epidermal replicas. The cement is still available and contains the same polymer, nitrocellulose, used more than a century ago, albeit in a bit less volatile solvent system. Inspection of the labels or material safety data sheets of the commonly used nail polishes reveals nitrocellulose as a primary component, along with some plasticizers. Nitrocellulose laquer is currently readily available in disposable aerosol spray canisters as furniture finish under the trade name Deft (Deft Inc., Irvine, CA, www.deftfinishes.com). It is an extremely rapid method of making very large stomatal peels and requires minimal equipment. We found it to be ideally suited to the tough broad flat glabrous sorghum leaves. However, the method suffers from the same disadvantages as the commonly encountered nail varnish method. The resulting peel is affixed to a piece of tape on which the adhesive is covered with laquer making mounting and archiving difficult.

The development of the CA/acetone system is similar to that of nitrocellulose laquer. The CA/acetone system was originally described as a varnish directly applied to the leaf surface (Long and Clements, 1934). The CA/acetone system yielded high resolution reproductions of the epidermal surface, and resulted in clearer mounts than nitrocellulose because it did not become translucent or milky in response to moisture next to stoma (the collodion method was thought to be able to measure transpiration rates). Cellulose acetate was later used as a thin film dissolved with acetone (Payne, 1970), then thicker strips softened with acetone (McDonald, 1977) and in this report as archivable plates softened with solvent and stored for later analysis. The method using CAB is simply an extension of the CA method using a closely related polymer, which is available in greater thickness at less expense. The CA is readily available in thicknesses up to 0.16 mm (1/16 in), while CAB is available in thicknesses up to 4.74 mm (3/16 in). The advantage of the CAB/acetone system is that very large, stable detailed replicas of epidermal surfaces can be produced with a readily available polymer and solvent of relatively low toxicity. Although it is not a difficult technique, it is more complex and time consuming to carry out than the spray laquer peel method, and might not be as well suited to the field.

The PMMA/$MeCl_2$ system produced acceptable epidermal impressions, at least for determining stomatal density, but we were unable to achieve the detail of the CAB/acetone system across an entire leaf surface. However, thinner slides could be made with less warping as compared to the CA/acetone system. The resulting PMMA slides were very stable and exhibited no apparent warping. Moreover, $MeCl_2$ dissolves epicuticular waxes so this might be the system of choice for waxy leaves. We had very limited success with the PVC/MEK system. While PVC/MEK resulted in stable slides and stoma could be visualized, the level of detail was simply not as great as with either the CAB/acetone or the PMMA/$MeCl_2$ systems, at least with bright field microscopy, perhaps because of the clarity of the resulting impressions. The use of phase contrast, or oblique illumination to view these impressions was not investigated.

CONCLUSION

Adaptation and extension of established methods for creating epidermal replicas for viewing under bright field microscopy yielded novel practical techniques for creating very large epidermal peels or castings across leaf surfaces. Since each polymer/solvent system exhibited unique strengths and weaknesses a general recommendation cannot be made, other than to try different methods to find the one most suitable for the application. The PMMA/$MeCl_2$ system and the nitrocellulose spray laquer systems have the advantage that the materials are very inexpensive and may be locally available, so they should probably be tried first. We preferred the CAB/acetone system because of the exquisite detail obtained, but it suffered from the drawback of requiring rather thick plates to prevent warping. Exploitation of other polymer and solvent combinations may yield additional improvements.

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